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Detection of GB Virus-C/Hepatitis G Virus Genome in Peripheral Blood Mononuclear Cells and Liver Tissue

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The replication site for the GB virus-C/hepatitis G virus (GBV-C/HGV) was investigated by using polymerase chain reaction (PCR)-based assays and in situ hybridisation. A total of 28 patients with consecutive GBV-C/HGV infection were enrolled in this study: Nine patients were being treated with immunosuppressive therapy after liver transplantation, and the remaining 19 patients were not receiving such treatment. GBV-C/HGV RNA was detected by using reverse transcriptase-polymerase chain reaction (RT-PCR) and was quantitated by using competitive RT-PCR in all patients. Positive and negative strands of GBV-C/HGV RNA in liver tissue were detected with in situ hybridisation by using RNA probes that were specific for the GBV-C/HGV genome. Concentrations of GBV-C/HGV RNA in serum were significantly higher ($P = 0.003$) in the nine patients who were receiving immunosuppression (median, 10^7 copy/ml; range, 10^5–10^7) than in the 19 patients who were not receiving immunosuppressive therapy (median, 10^4 copy/ml; range, 10^2–10^7). In situ hybridisation of GBV-C/HGV RNA was performed on paraffin-embedded liver tissue that was obtained from six patients with GBV-C/HGV infection. Two of those six patients were receiving immunosuppressive therapy, and four were not. Significant positive signals were observed in the samples from two of the six patients who were infected with GBV-C/HGV, but such signals were not observed in any of the six patients who were without the infection. The two patients with positive signals (both were undergoing immunosuppressive therapy) showed both positive and negative strands of GBV-C/HGV RNA in mononuclear cells that infiltrated into portal areas, but neither of the strands was observed in hepatocytes. Moreover, the GBV-C/HGV replication was analysed in peripheral blood mononuclear cells by using strand-specific PCR (conventional RT-PCR and rTth method). Two of the six patients were positive for negative-strand GBV-C/HGV RNA by using conventional RT-PCR. In conclusion, GBV-C/HGV replication was active under an immunosuppressive state, and it is suggested that GBV-C/HGV replicates in mononuclear cells. ***J. Med. Virol. 57:114–121, 1999.***

KEY WORDS: hepatitis virus; GB virus-C/hepatitis G virus; strand-specific polymerase chain reaction; in situ hybridisation

INTRODUCTION

GB virus C (GBV-C) and hepatitis G virus (HGV) have been identified recently as possible causative agents for non-A, non-B, and non-C hepatitis [Simons et al., 1995; Linnen et al., 1996]. The two viruses have almost identical nucleotide sequences and are now considered to be different isolates of the same virus in the *Flaviviridae* family [Alter, 1996]. Because the nomenclature of the new virus has not been determined, the term GBV-C/HGV is used in this paper. GBV-C/HGV is related to the hepatitis C virus (HCV) in its genomic structure, and, like HCV, it is transmissible by blood transfusion and has a tendency to develop a chronic carrier state [Aikawa et al., 1996; Linnen et al., 1996; Schmidt et al., 1996].

The pathogenicity of GBV-C/HGV as a hepatitis virus usually has been reported as negligible or weak

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TABLE I. Features of Six Patients who Underwent Histologic Examination*

Patient	Age (years)	Gender	AST/ALT	Immunosuppressive therapy	Infected viruses	Liver histology
1	50	F	41/72	tacrolimus, PSL	GBV-C/HGV	Recurrence of PBC
2	43	F	39/47	cyclosporin A, PSL	GBV-C/HGV	Recurrence of PBC
3	48	M	69/97	(–)	HCV, GBV-C/HGV	Chronic hepatitis
4	51	F	35/51	(–)	HCV, GBV-C/HGV	Chronic hepatitis
5	46	F	81/87	(–)	GBV-C/HGV	Chronic hepatitis
6	52	F	35/51	(–)	GBV-C/HGV	Chronic hepatitis

*AST/ALT, asparate aminotransferase/alanine aminotransferase; PSL, prednisolone; GBV-C/HGV, GB virus C/hepatitis G virus; HCV, hepatitis C virus; PBC, primary biliary cirrhosis.

[Berenguer et al., 1996; Tanaka et al., 1996; Cotler et al., 1997]. Although several investigators reported an association between GBV-C/HGV infections and the occurrence of fulminant hepatitis [Yoshiba et al., 1995; Heringlake et al., 1996], others did not confirm this relationship [Hwant et al., 1997; Kanda et al., 1997]. Thus, the issue of GBV-C/HGV replication sites has been raised to address the clinical significance of GBV-C/HGV. Madejon et al. [1997] reported that GBV-C/HGV was a hepatotropic virus that replicates in the human liver. However, there are several reports showing that the liver was not the primary replication site for GBV-C/HGV [Laskus et al., 1997a,b; Mellor et al., 1998; Pessoa et al., 1998]. To determine the replication sites, both groups relied on strand-specific detection of the GBV-C/HGV genome with polymerase chain reaction (PCR). The specificity of detecting strand-specific viral genomes with PCR has been improved, but this method still does not produce definitive results.

In the present study, GBV-C/HGV replication sites were analysed by in situ hybridisation in liver tissue obtained not only from immunocompetent patients but also from immunosuppressed patients who were assumed to have higher GBV-C/HGV replication states.

MATERIALS AND METHODS

Patients

A total of 28 patients who were serum-positive for GBV-C/HGV RNA were enrolled in the present study. These patients were selected from 347 patients who were seen at Shinshu University Hospital between October, 1996 and April, 1997 who consented to the measurement of GBV-C/HGV RNA. Of the 28 patients, nine were receiving immunosuppressive therapy after orthotopic liver transplantation. All 28 patients were positive for GBV-C/HGV RNA at least twice within intervals of more than 3 months.

The nine patients with immunosuppressive therapy consisted of two males and seven females ranging in age from 2 years to 50 years. The immunosuppressive agents that were administered to patients were as follows: tacrolimus in six patients (trough levels, 5–10 ng/ml), cyclosporin A in three patients (trough levels, 100–150 ng/ml), and azathioprine in two patients. Prednisolone (5–10 mg/day) was also used in all nine patients. Causal diseases requiring liver transplantation were congenital bile duct obstruction in five patients, primary biliary cirrhosis in two patients, Alagille syndrome in one patient, and familial amyloid polyneuropathy in one patient. The duration of immunosuppressive therapy was 8–51 months (mean, 27 months). No patient had concomitant hepatitis B virus (HBV) or HCV infection.

The 19 patients who did not undergo immunosuppressive therapy were seen in our hospital because of chronic hepatitis; four patients presented with hepatitis B, 13 patients presented with hepatitis C, and two patients presented with non-B/non-C hepatitis. Ten males and nine females with an age range from 35 years to 71 years were in this group. Serum samples were stored at −70°C until the measurements were taken.

Liver Tissues

Histological localisation of GBV-C/HGV was analysed by in situ hybridisation on six paraffin-embedded liver specimens obtained from six patients during the period of the patients' selection. Among six patients with GBV-C/HGV RNA, two patients underwent immunosuppressive therapy (both were transplanted due to primary biliary cirrhosis), and four patients were not given immunosuppressive therapy (two patients had chronic hepatitis C and the other had chronic non-B/non-C hepatitis). The histological diagnoses shown in Table I were based on established criteria [Desmet et al., 1994] and were confirmed by histologic review by an independent observer. Six chronically diseased liver specimens showing no evidence of GBV-C/HGV infection were used as controls: These samples were taken during the same period as the GBV-C/HGV RNA samples. Two patients in the control group had chronic hepatitis B, two patients had chronic hepatitis C, and two patients were diagnosed with primary biliary cirrhosis.

Virological Assays

Hepatitis B surface antigen and hepatitis C antibody were tested by using commercially available enzyme-linked immunosorbent assay kits (International Reagents Corporation, Kobe, Japan). HCV RNA was tested by nested reverse transcribed (RT)-PCR using a method described previously [Tanaka et al., 1993].

Detection and Quantitative Measurement of GBV-C/HGV RNA

GBV-C/HGV RNA in the serum was detected and quantitated by nested PCR using primers derived from the 5′-untranslated region (UTR) of the GBV-C/HGV

genome. Two sets of nested primers were used, including the outer primer pair of 299 base span (sense: U1, 5′-GGCCAAAAGGTGGTGGATGGGTGA-3′; antisense: U4, 5′-CCACTGGTCCTTGTCAACTCGC-3′) and the inner primer pair of 210 base span (sense: U2, 5′-GGTTGGTAGGTCGTAAATCCCGGT-3′; antisense: U3, 5′-GACGTGGACCGTACGTGGGC-3′). Total RNA was extracted from 100 μl of serum sample by using an ISOGEN LS kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Purified RNA was resuspended in 15 μl of distilled water. For reverse transcription, all extracted RNA solution was used in 20 μl of final mixture consisting of 1.25 μmol/litre antisense primer (U4), 30 units of Moloney murine leukaemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD), 1 × PCR buffer (TaKaRa Biomedicals, Kyoto, Japan), 200 μmol/litre dNTP (TaKaRa Biomedicals), and 15 units of recombinant RNAsin (Promega, Madison, WI). Reverse transcription was undertaken for 60 minutes at 42°C, and the resultant cDNA preparation was added to 80 μl PCR solution containing 1 × PCR buffer (TaKaRa Biomedicals), 200 μmol/litre dNTP, 0.25 μmol/litre sense primer (U1), and 3 units of *Taq* polymerase (TaKaRa Biomedicals) in a final volume of 100 μl. After initial denaturation at 94°C for 3 minutes, 35 cycles of first amplification were carried out at 94°C for 30 seconds, at 56°C for 30 seconds, and at 72°C for 30 seconds, followed by a 5-minute extension at 72°C. Then, 2 μl of the first PCR product were used for the second PCR with a set of inner primers in 50 μl of PCR solution containing 1 × PCR buffer (TaKaRa Biomedicals), 200 μmol/litre dNTP, 0.5 μmol/litre sense primer (U2), 0.5 μmol/litre antisense primer (U3), and 2.5 units of *Taq* polymerase (TaKaRa Biomedicals). Second PCR cycles were undertaken in the same manner as the first PCR round. The expected 210 base pairs were detected by electrophoresis on a 3.0% agarose gel and stained by ethidine bromide.

The amount of GBV-C/HGV RNA was measured by a competitive RT-PCR assay using a DNA competitor. We raised a mutant GBV-C/HGV cDNA as a competitor by introducing an internal deletion of 36 base pairs into the wild GBV-C/HGV cDNA generated by the pGEM-T vector system (Promega). The competitor fragment was diluted serially in distilled water to yield three different concentrations: 10 copy/10 μl, 10^3 copy/10 μl, and 10^5 copy/10 μl. Total RNA was extracted from 200 μl of the serum sample, and reverse transcription was undertaken in the same manner as the detection of GBV-C/HGV RNA. One microlitre of cDNA sample (corresponding to RT products from 10 μl of serum sample) was mixed with 10 μl of each competitor at three concentrations, and nested PCR amplification was carried out in the same manner as that used for detecting GBV-C/HGV RNA. The amount of GBV-C/HGV RNA was determined from electrophoretic patterns of the amplified GBV-C/HGV genome and its competitor. The amount was then expressed as copy per millilitre (Fig. 1).

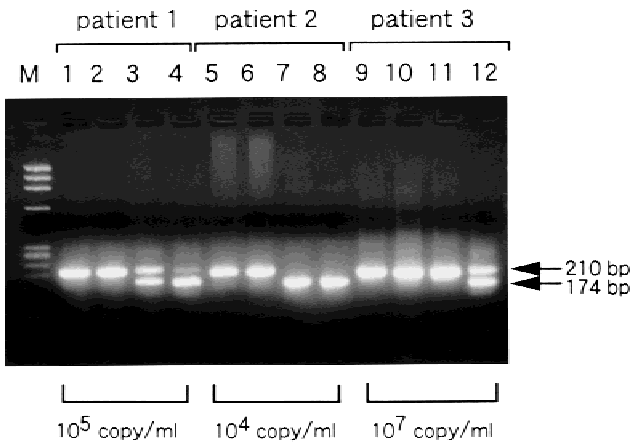

Fig. 1. Quantification of GB virus C/hepatitis G virus (GBV-C/HGV) RNA concentration by a competitive titer. Varying amounts of the competitor (10 copy/10 μl, 10^3 copy/10 μl, 10^5 copy/10 μl) were coamplified with a constant of cDNA (corresponding to product derived from 10 μl of serum). In lanes 1, 5, and 9, competitor was not added, and 10 μl of reverse transcriptase (RT) product were added to the first polymerase chain reaction (PCR) buffer. In lanes 2, 6, and 10, competitor (10 copy/10 μl) was coamplified. In lanes 3, 7, and 11, competitor (10^3 copy/10 μl) was coamplified. In lanes 4, 8, and 12, competitor (10^5 copy/10 μl) was coamplified. If a lane was positive for both bands, then the concentrations of sample cDNA were similar to those of competitor DNA. In patients 1 and 3, because lanes 3 and 12 were positive for both bands, the concentrations of patients 1 and 3 were regarded to be 10^3 copy/10 μl and 10^5 copy/10 μl, respectively. In patient 2, lanes 5 and 6 were positive for sample cDNA, and lanes 7 and 8 were positive for competitor DNA. The serum concentration of patient 2 was midway between the concentrations of 10^1 copy/10 μl and 10^3 copy/10 μl; thus, it was regarded to be 10^2 copy/10 μl. Because the concentrations of serum GBV-C/HGV RNA were expressed as copy per millilitre in this paper, the serum concentrations of patients 1, 2, and 3 were 10^5 copy/ml, 10^4 copy/ml, and 10^7 copy/ml, respectively. M, marker 4 (Wako, Osaka, Japan).

Preparation of RNA Probe for GBV-C/HGV RNA

GBV-C/HGV RNA was isolated from 100 μl of serum from a patient with chronic GBV-C/HGV infection by using an ISOGEN LS kit (Nippon Gene) according to the manufacturer's instruction. A single-stranded cDNA was then synthesised by reverse transcription of the extracted RNA by using a primer, K-4 (5′-CGAATGAGTCAGAGGACGGGGTATCCTCCT-3′). By using this cDNA as a template, a GBV-C/HGV-specific nucleotide sequence (NS5 of GBV-C/HGV; from +6,904 to +7,059 according to the published sequence; Genebank accession no. U44402) was amplified by PCR using an upstream primer, K-1 (5′-CTCTTTGTGGTAGTAGCCGAGAGATGCCTGTGT-3′), and the K-4 primer as a downstream primer. Fifty microlitres of the reaction mixture containing 1 × PCR buffer, 200 μmol/litre dNTP, 1 μmol/litre of each primer, and 1.25 units of *Taq* polymerase were subjected to PCR consisting of 30 cycles at 94°C for 1 minute, at 55°C for 1 minute, and at 72°C for 1 minute. The amplified product was then subcloned between the *Sac* II and *Spe* I sites of the pGEM-T vector (Promega). Nucleotide sequence of the resultant vector revealed that 5′ end of the introduced NS5 faced the *Sac* II site of pGEM-T; thus, an antisense RNA probe was obtained by using an *Nco* I-cut template and SP6 RNA polymerase with a DIG RNA

labelling kit (Boehringer Mannheim, Mannheim, Germany). Similarly, a digoxigenin-labelled sense probe was prepared by using a *Sal* I-cut template and T7 RNA polymerase with the same kit. The antisense and sense probes were used for the detection of positive and negative strands of the GBV-C/HGV RNA, respectively.

In Situ Hybridisation of GBV-C/HGV RNA

Tissue specimens were subjected to in situ hybridisation to detect GBV-C/HGV RNA by using a nonradioactive detection system [Kawakami and Nakayama, 1997]. After the tissue sections were deparafinised in xylene, the hydrated slides were immersed in 0.2 mol/litre HCl for 20 minutes and then digested with 100 μg/ml proteinase K at 37°C for 20 minutes, followed by postfixation with 4% paraformaldehyde. These slides were rinsed with 2 mg/ml glycine and subsequently acetylated for 10 minutes in freshly prepared 0.25% acetic anhydride in 0.1 mol/litre triethanolamine, pH 8.0. The hydrated slides were then defatted with chloroform and then air dried. After prehybridisation with 50% deionized formamide/2 × standard saline citrate (SSC) for 1 hour at 45°C, the slides were hybridised with 0.5 mg/ml of the antisense or sense probe in 50% deionized formamide; 2.5 mmol/litre EDTA, pH 8.0; 300 mmol/L NaCl; 1 × Denhardt's solution; 10% dextran sulphate; and 1 mg/ml Brewer's yeast tRNA at 45°C for 16 hours.

After hybridisation, the slides were washed in 50% formamide/2 × SSC for 1 hour at 45°C and digested with 10 μg/ml RNase A at 37°C for 30 minutes. After washing with 2 × SSC/50% formamide at 45°C for 1 hour, 1 × SSC/50% formamide at 45°C for 1 hour, and 1 × SSC/50% formamide at room temperature for 30 minutes, the sections were subjected to immunohistochemistry for detection of the hybridised probes by using an alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim). The alkaline phosphatase reaction was visualised with nitroblue tetrazolium and 5-bromo-O-4-chloro-3-indolylphosate.

Immunohistochemistry

To determine the characteristics of the mononuclear cells infiltrating into the portal area, the serial sections of the liver tissues were also subjected to immunohistochemistry by using monoclonal antibodies, L26, UCHL-1, and KP-1. These antisera are specific for CD20 (pan-B cell), CD45RO (pan-T cell), and CD 68 (macrophage), respectively, and were obtained from DAKO (Glostrup, Denmark). The immunohistochemical detection was carried out by using the indirect method followed by counterstaining with haematoxylin, as described previously [Yoshizawa et al., 1993]. A control study was done by omitting the primary antibody from the staining procedure, and no specific staining was found.

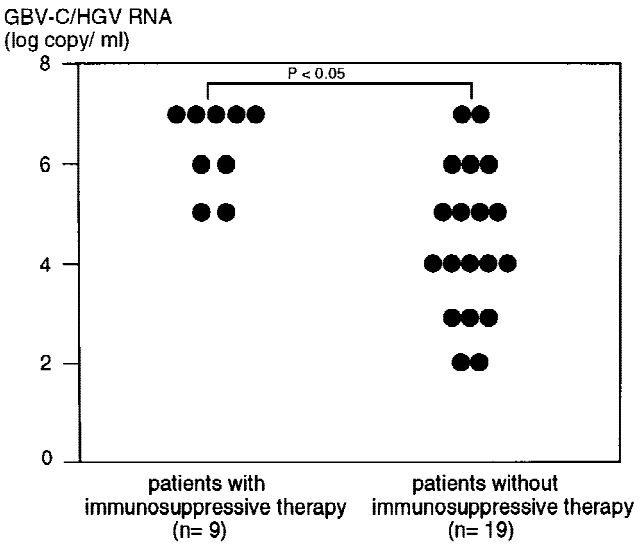

Fig. 2. Comparison of GBV-C/HGV RNA concentration in serum between patients with and without immunosuppressive therapy. GBV-C/HGV RNA concentration was significantly higher ($P = 0.003$) in patients with immunosuppressive therapy (median, 10^7 copy/ ml; range, 10^5–10^7) than in patients without immunosuppressive therapy (median, 10^4 copy/ ml; range, 10^2–10^7).

Strand-Specific Detection of GBV-C/HGV RNA in Peripheral Blood Mononuclear Cells

We investigated the GBV-C/HGV genome in peripheral blood mononuclear cells (PBMCs) of six patients whose liver tissues were analysed for the genome by in situ hybridisation. PBMCs were prepared from 10 ml of whole blood by using a standard procedure of Ficoll-Hypaque gradient centrifugation. Collected PBMCs were washed three times with phosphate-buffered saline and then frozen at −70°C until measurement. RNA was extracted from the PBMCs by using an ISOGEN kit (Nippon Gene).

Strand-specific detection of GBV-C/HGV RNA was carried out by using conventional RT-PCR [Saleh et al., 1994; Chang et al., 1996] and rTth methods [Laskus et al., 1997a,b]. Conventional RT-PCR was performed under the same conditions used for detecting GBV-C/HGV RNA in serum with the following exceptions: In RT, only a sense primer (U1) was used for detecting the negative strand, and only an antisense primer (U4) was used for detecting the positive strand. In the detection of the negative strand, the procedure was augmented with the following arrangement: Products of RT were boiled in a water bath for 30 minutes to inactivate reverse trancriptase activity, and they were then immediately chilled on ice. Subsequently, the samples were treated with RNase A (Boehringer Mannheim) at a concentration of 50 μg/ml (30 minutes at 37°C), as described previously [Saleh et al., 1994; Chang et al., 1996].

To support the specificity of the strand-specific detection of GBV-C/HGV RNA, four kinds of negative control studies were carried out, resulting in no sample, no reverse transcriptase, no primer in the RT

TABLE II. Strand-Specific Detection of GBV-C/HGV RNA in Liver Tissue and Peripheral Blood Mononuclear Cells*

Patient	GBV-C/HGV RNA in serum (copy/ml)	PBMCs (positive/negative strand)		Liver tissue (positive/negative strand)
		Conventional RT-PCR	rTth method	In situ hybridisation
1	10^7	+/+	+/–	+/+
2	10^7	+/+	+/–	+/+
3	10^5	+/–	+/–	–/–
4	10^3	+/–	–/–	–/–
5	10^7	+/–	–/–	–/–
6	10^4	–/–	–/–	–/–

*GBV-C/HGV, GB virus C/hepatitis G virus; PBMCs, peripheral blood mononuclear cells; RT-PCR, reverse transcriptase-polymerase chain reaction; rTth, recombinant Thermus thermophilus.

reaction, and no primer in the first PCR reaction. Consequently, all negative controls proved to be negative in each assay.

Tth-based RT-PCR was undertaken by using a Thermostable rTth reverse transcriptase RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). The cDNA was generated in 20 μl of reaction mixture containing 1.25 μmol/litre of a primer (U1 for detecting negative strand, U4 for detecting positive strand), 1 × RT buffer, 1 mM $MnCl_2$, 200 μmol/litre of dNTP, and 5 units of rTth polymerase. After incubation for 15 minutes at 70°C, 80 μl of PCR master mix, which contained 0.30 μmol/litre of a primer (U4 for detecting negative strand, U1 for detecting positive strand), 1 × chelating buffer, and 2.5 mol/litre of $MgCl_2$, were added to the RT reaction mixture. The amplification was performed in a Perkin Elmer GeneAmp PCR System 9600 thermalcycler as follows: initial denaturing for 1 minute at 94°C and then 50 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds followed by a final extension at 72°C for 5 minutes. Then, 2 μl of PCR product were subjected to second PCR with the set of inner primers used in the conventional PCR method. PCR products were analysed by gel electrophoresis.

Statistical Analysis

Statistical analysis was carried out by using the Mann-Whitney test. A significant level was set at $P = 0.05$.

RESULTS

GBV-C/HGV RNA Concentration in Serum

The concentration of GBV-C/HGV RNA in serum was distributed between 10^2 and 10^7 copy/ml by using competitive RT-PCR. The concentration in nine patients with immunosuppressive therapy (median, 10^7; range 10^5–10^7 copy/ml) was significantly higher ($P = 0.003$, Mann-Whitney test) than that in 19 patients without the therapy (median, 10^4; range, 10^2–10^7 copy/ml) (Fig. 2).

Detection of GBV-C/HGV RNA by In Situ Hybridisation

A significant amount of the signal for GBV-C/HGV RNA by in situ hybridisation was observed in two of the six patients with GBV-C/HGV viremia (Table II); no hybridisation signal was detected in the 6 control patients without GBV-C/HGV viremia. Both patients with the positive signal were under immunosuppressive therapy, but the remaining four patients were not given such therapy. In the two patients showing positive signals, both positive and negative strands of GBV-C/HGV RNA were stained in the cytoplasm of mononuclear cells infiltrated in the portal area, and the positive cells observed in using antisense or sense probes were almost identical (Fig. 3B,C). No significant signal was observed in other parts of the liver tissue, including the hepatocytes.

To characterise further the GBV-C/HGV-infected mononuclear cells infiltrating in the portal area, immunohistochemistry was carried out on serial sections of the liver tissues by using mononuclear antibodies against pan-B (L26), pan T (UCHL-1), and macrophages (KP-1). Figure 3 shows that the GBV-C/HGV-infected mononuclear cells also seemed to be verified by UCHL-1 or KP-1 antibodies, suggesting that GBV-C/HGV replicates primarily in the T cells or macrophages.

Detection of GBV-C/HGV RNA in PBMCs

The results of strand-specific detection of GBV-C/HGV RNA in PBMCs are shown in Table II. The negative strand was detected by the conventional RT-PCR method in two patients (patients 1 and 2) who were positive for it in mononuclear cells infiltrated in the liver by in situ hybridisation. On the other hand, the negative strand was not detected by the rTth method in all patients. The positive strand was detected in five patients by using the conventional RT-PCR method and in three patients by using the rTth method.

DISCUSSION

The present study showed that GBV-C/HGV concentration in serum was significantly higher in patients with immunosuppressive therapy than in those without the therapy. A similar tendency has been reported in the serum concentration of HCV RNA, in that the concentrations increased as a result of immunosuppressive therapy [Chazouilleres et al., 1994; Gane et al., 1996]. These results suggest that a replication not

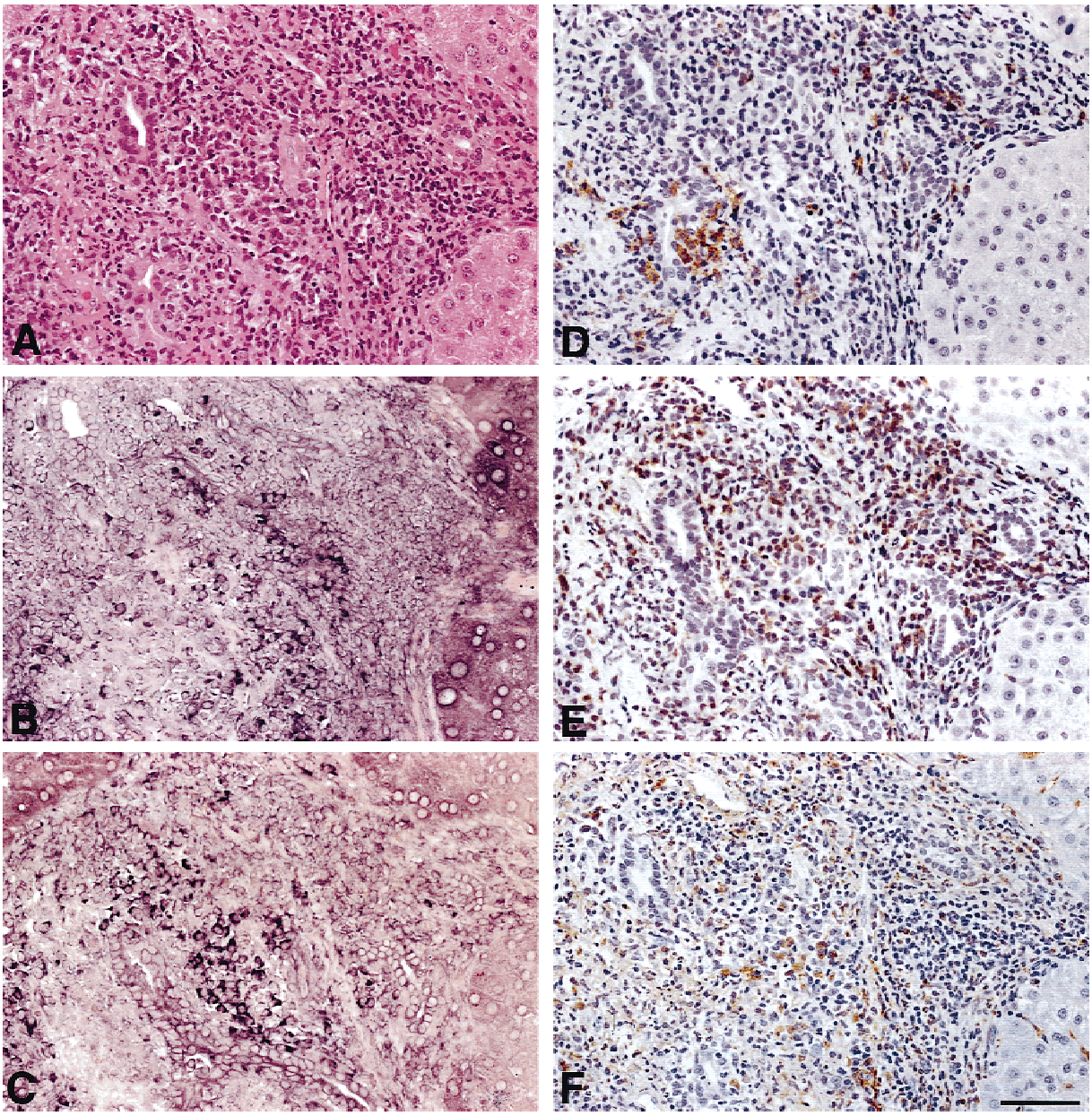

Fig. 3. **A–F:** Identification of GBV-C/HGV RNA in liver tissue. Marked infiltration of mononuclear cells in the portal area is evident in the liver biopsy specimen obtained from patient 1 (see Table I) 51 months after liver transplantation (A; hematoxylin and eosin). Positive signals for GBV-C/HGV are detectable in the mononuclear cells in the portal area but are not detectable in the hepatocytes (B, antisense probe; C, sense probe). Immunohistochemistry reveals that most of the GBV-C/HGV-infected cells in the portal area are likely to be T cells or macrophages. D: Monoclonal antibody against pan-B (L26). E: Monoclonal antibody against pan-T (UCHL-1). F: Monoclonal antibody against macrophages (KP-1). Scale bar = 100 μm.

only of HCV but also of GBV-C/HGV was suppressed by a host immune system; thus, replications of the viruses could be accelerated under immunosuppressive states.

There remains the question whether or not GBV-C/HGV is hepatotropic. To address this question, several investigators analysed the replication site of GBV-C/HGV by detecting the negative strand of the GBV-C/HGV genome. Several methods using PCR techniques have been reported on strand-specific detection of viral genomes, including those using conventional RT-PCR [Saleh et al., 1994; Chang et al., 1996; Kao et al., 1997], tagged RT-PCR [Lanford et al., 1994], and rTth methods [Young et al., 1993; Lanford et al., 1994, 1995]. However, these methods have a limitation for exact detection of a negative strand. The conventional RT-PCR method increases the risk of obtaining false-positive results because of false priming or self priming of the positive strand [McGuinness et al., 1994; Kao et al., 1997]. Although there remains a risk of false positives when using tagged RT-PCR and rTth methods, this risks is reduced substantially compared with that incurred when using the conventional RT-PCR. With

the rTth method, an incorrect strand is detected by a lapse of strand specificity at levels 10,000-fold higher than are required to detect the correct strand of RNA [Lanford et al., 1995]. Similarly, false signals for the negative strand of RNA can be obtained by using the tagged RT-PCR method if sufficient quantities of positive-strand RNA are present [Lerat et al., 1996]. Furthermore, because the tagged RT-PCR and rTth methods are 10–100 times less sensitive than conventional RT-PCR, the former methods incur a higher risk of producing false negatives [Laskus et al., 1997a; Mellor et al. [1998]. Indeed, results concerning the GBV-C/HGV replication site have been controversial. Madejon et al. [1997] reported that the negative strand of the GBV-C/HGV genome could be detected in the liver of patients with GBV-C/HGV infection by using tagged RT-PCR. In contrast, Laskus et al. [1997a,b] reported that the negative strand could not be found in the liver by using the rTth method, and they concluded that the liver was not the primary replication site for GBV-C/HGV. Therefore, it is necessary to adopt techniques other than PCR when studying the replication site of GBV-C/HGV.

In the present study, in situ hybridisation assay detected the positive and negative strands of GBV-C/HGV RNA separately. The assay is based on hybridisation with strand-specific RNA probes, which are transcribed from a cloned cDNA. Thus, it is unlikely that this assay will produce the false positives that have been obtained by methods using PCR. Indeed, the present study detected no positive signals in the liver tissues of any of the patients without GBV-C/HGV infection.

By using in situ hybridisation, positive signals of GBV-C/HGV RNA were detected in the liver tissues in two of the six patients with GBV-C/HGV infection. Both patients with positive signals were treated with immunosuppressive therapy, and they had high serum concentrations of GBV-C/HGV RNA (10^7 copy/ml). The remaining four patients who showed no positive signals did not receive immunosuppressive therapy. It is possible that the active replication of GBV-C/HGV due to immunosuppressive therapy resulted in a successful detection of the GBV-C/HGV genome by in situ hybridisation. In the two patients with positive signals, both positive and negative strands of GBV-C/HGV RNA were detected in mononuclear cells that infiltrated in the portal area. However, no signal was observed in other cells, including hepatocytes. It has been reported that most of *Flaviviridae*, including HCV, can replicate in mononuclear cells [Saleh et al., 1994; Kao et al., 1997]. These results indicate that GBV-C/HGV replicates not in hepatocytes but in mononuclear cells. Furthermore, our immunohistochemical study suggests that such mononuclear cells, for the most part, are T lymphocytes or macrophages.

To confirm the replication of GBV-C/HGV in mononuclear cells, PBMCs were investigated by using the strand-specific RT-PCR method. In the two patients who showed positive signals with in situ hybridisation, both positive and negative strands of GBV-C/HGV RNA were found in the PBMCs by using the conventional RT-PCR. However, in those patients, only the positive strand could be detected by using the rTth method. The results obtained with conventional RT-PCR can be false positives. Nevertheless, this further supports the results obtained with in situ hybridisation. In conclusion, GBV-C/HGV replication was more active when it was accompanied by immunosuppressive therapy. GBV-C/HGV can replicate in mononuclear cells, and it is suggested that hepatocytes are not the primary site for GBV-C/HGV replication.

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REFERENCES

Aikawa T, Sugai Y, Okamoto H. 1996. Hepatitis G infection in drug abusers with chronic hepatitis C. N Engl J Med 334:195–196.

Alter HJ. 1996. The cloning and clinical implications of HGV and HGBV-C. N Engl J Med 334:1536–1537.

Berenguer M, Terrault NA, Piatak M, Yun A, Kim JP, Lau JYN, Lake JR, Roberts JR, Ascher NL, Ferrell L, Wright TL. 1996. Hepatitis G virus infection in patients with hepatitis C virus infection undergoing liver transplantation. Gastroenterology 111:1569–1575.

Chang TT, Young KC, Yang YJ, Lei HY, Wu HL. 1996. Hepatitis C virus RNA in peripheral blood mononuclear cells: comparing acute and chronic hepatitis C virus infection. Hepatology 23:977–981.

Chazouilleres O, Kim M, Coombes C, Ferrell L, Bacchetti P, Roberts J, Ascher NL, Neuwald P, Wilber J, Urdea M, Quan S, Sanchez-Pescador R, Wright TL. 1994. Quantitation of hepatitis C virus RNA in liver transplant recipients. Gastroenterology 106:994–999.

Cotler SJ, Gretch DR, Bronner MP, Tateyama H, Emond MJ, dela Rosa C, Perkins JD, Chrithers RL, Jr.. 1997. Hepatitis G virus co-infection dose not alter the course of recurrent hepatitis C virus infection in liver transplantation recipients. Hepatology 26:432–436.

Desmet VJ, Gerber M, Hoofnagele JH, Manns M, Scheuer PJ. 1994. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 19:1513–1520.

Gane EJ, Naoumov NV, Qian KP, Mondelli MU, Maertens G, Portmann BC, Lau JYN, Williams R. 1996. A longitudinal analysis of hepatitis C virus replication following liver transplantation. Gastroenterology 110:167–177.

Heringlake S, Osterkamp S, Trautwein C, Tillmann HL, Boker K, Muerhoff S, Mushahwar G, Hunsmann G, Manns MP. 1996. Association between fulminant hepatic failure and a strain of GBV virus C. Lancet 348:1626–1629.

Hwang SJ, Lu RH, Chan CY, Wang YJ, Wu JC, Lee SD. 1997. The role of hepatitis G virus infection in patients with acute posttransfusion hepatitis in Taiwan. Gastroenterology 112:1260–1264.

Kanda T, Yokosuka O, Ehata T, Maru Y, Imazeki F, Saisho H, Shiratori Y, Omata M. 1997. Detection of GBV-C RNA in patients with non A-E fulminant hepatitis by reverse-transcription polymerase chain reaction. Hepatology 25:1261–1265.

Kao JH, Chen PJ, Lai MY, Wang TH, Chen DS. 1997. Positive and negative strand of hepatitis C virus RNA sequences in peripheral blood mononuclear cells in patients with chronic hepatitis C: no correlation with viral genotypes 1b, 2a, and 2b. J Med Virol 52: 270–274.

Kawakami M, Nakayama J. 1997. Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by in situ hybridization. Cancer Res 57:2321–2324.

Lanford RE, Sureau C, Jacob JR, White R, Fuers TR. 1994. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. Virology 205:606–614.

Lanford RE, Chavez D, Chisari FV, Sureau C. 1995. Lack of detection

of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. J Virol 69:8079–8083.

Laskus T, Radowski M, Wang LF, Vargas H, Pakela J. 1997a. Lack of evidence for hepatitis G virus replication in the livers of patients coinfected with hepatitis C and G viruses. J Virol 71:7804–7806.

Laskus T, Wang LF, Radowski M, Jang SJ, Vargas H, Dodson F, Fung J, Rakela J. 1997b. Hepatitis G virus infection in American patients with cryptogenic cirrhosis: no evidence for liver replication. J Infect Dis 176:1491–1495.

Lerat H, Berby F, Trabaud MA, Vidalin O, Major M, Trepo C, Inchauspe G. 1996. Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J Clin Invest 97:845–851.

Linnen J, Wages J, Jr., Zhang-Keck Z-Y, Fry KE, Krawczynski KZ, Alter HJ, Koonin E, Gallagher M, Alter MJ, Hadziyannis S, Karayiannis P, Fung K, Nakatsuji Y, Shih JW-K, Young L, Piatak M Jr, Hoover C, Fernandez J, Chen S, Zou J-C, Morris T, Hyama KC, Ismay S, Lifson JD, Hess G, Foung SKH, Thomas H, Bradley D, Margolois H, Kim JP. 1996. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. Science 271:505–508.

Madejon A, Fogeda M, Bartolome J, Pardo M, Gonzalez C, Cotonat T, Carreno V. 1997. GB virus C RNA in serum, liver, and peripheral blood mononuclear cells from patients with chronic hepatitis B, C, and D. Gastroenterology 113:573–578.

McGuinness PH, Bishop GA, McCaughn GW, Trowbridge R, Gowans EJ. 1994. False detection of negative-strand hepatitis C virus RNA. Lancet 343:551–552.

Mellor J, Haydon G, Blair C, Livingstone W, Simmonds P. 1998. Low level or absent in vivo replication of hepatitis C virus and hepatitis G virus/GB virus C in peripheral blood mononuclear cells. J Gen Virol 79:705–714.

Pessoa MG, Terrault NA, Detmer J, Kolberg J, Collins M, Hassoba HM, Wright TL. 1998. Quantitation of hepatitis G and C viruses in the liver: evidence that hepatitis G virus is not hepatotropic. Hepatology 27:877–880.

Saleh MG, Tibbs CJ, Koskinas TJ, Pereira LMMB, Bomford AB, Portmann BC, Mcfarlane IG, Williams R. 1994. Hepatic and extrahepatic hepatitis C virus replication in relation to response to interferon therapy. Hepatology 20:1399–1404.

Schmidt B, Korn K, Fleckebnstein B. 1996. Molecular evidence for transmission of hepatitis G virus by blood transmission. Lancet 347:909.

Simons JN, Leary TP, Dawson GJ, Pilot- Matias TJ, Muerhoff AS, Schlauder GG, Desai SM, Mushahwar IK. 1995. Isolation of novel virus-like sequences associated with human hepatitis. Nature Med 1:564–569.

Tanaka E, Kiyosawa K, Nakatsuji Y, Inoue Y, Miyamura T, Chiba J, Furuta S. 1993. Clinical significance of antibodies to nonstructual and core proteins of hepatitis C virus in posttransfusion hepatitis patients during long-term follow-up. J Med Virol 39:318–324.

Tanaka E, Alter HJ, Nakatsuji Y, Shih JWK, Kim JP, Matsumoto A, Kobayashi M, Kiyosawa K. 1996. Effect of hepatitis G virus infection on chronic hepatitis C. Ann Int Med 125:740–743.

Yoshiba M, Okamoto H, Mishiro S. 1995. Detection of the GBV-C hepatitis virus genome in serum from patients with fulminant hepatitis of unknown aetiology. Lancet 346:1131–1132.

Yoshizawa K, Kiyosawa K, Yamada S, Furuta K, Yabu K, Kitano K, Akamatsu T, Nakayama J, Katsuyama T, Matsunami H, Kawasaki S, Makuuchi M, Nanba K, Furuta S. 1993. Establishment of Epstein-Barr virus-associated lymphoma call line SUBL with t(2;3)(p11;q27) from a liver transplant patient. Cancer Genet Cyogenet 71:155–163.

Young KK, Resnick RM, Myers TW. 1993. Detection of hepatitis C virus RNA by a combined reverse transcription polymerase chain reaction assay. J Clin Microbiol 31:882–886.